Reduction in dietary lysine increases muscle free amino acids through changes in protein metabolism in chickens

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ABSTRACT Taste is crucial to meat quality, and free Glu is an important taste-active component in meat. Our recent study showed that the short-term feeding of a low-Lys diet increases the concentration of free Glu and other free amino acids in chicken muscle and improves its taste. Here, we investigated the mechanisms by which the feeding of a low-Lys diet increases free Glu in chicken muscle. Two groups (n = 10 per group) of 28-day-old female Ross strain broiler chickens were fed diets with a graded Lys content of 90% or 100% of the recommended Lys requirement (according to National Research Council [1994] guidelines) for 10 D. Free amino acid concentrations and the mRNA abundance of protein metabolism–related genes were measured in breast muscle, and breast muscle metabolome analysis was conducted. Free Glu in muscle was increased by 51.8% in the Lys 90% group compared with the Lys 100% group ($P < 0.01$). Free threonine, glutamine, glycine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, and 3-methyl-histidine concentrations in breast muscle were also increased in the Lys 90% group ($P < 0.05$). Metabolome analysis also showed that free amino acids were increased in the Lys 90% group. The mRNA abundance of μ-calpain, caspase-3, and 20S proteasome C2 subunit were increased in the Lys 90% group ($P < 0.05$). Moreover, the free Glu concentration in muscle was correlated with mRNA abundance of μ-calpain ($r = 0.74$, $P < 0.01$), caspase 3 ($r = 0.69$, $P < 0.01$), 20S proteasome C2 subunit ($r = 0.65$, $P < 0.01$), and cathepsin B ($r = 0.52$, $P < 0.05$). Our study suggests that the feeding of a low-Lys diet to chickens increased the free Glu content of breast muscle by promoting protein degradation.

Key words: broiler, lysine, glutamate, free amino acid, protein metabolism

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INTRODUCTION

The essential amino acid Lys is often the limiting dietary amino acid in farm animals that consume predominantly cereal grain–based diets (National Research Council, 1989). Accordingly, most studies of the effects of dietary Lys on farm animals have focused on feed efficiency, growth performance, and meat yield (Kendall et al., 2008; Dozier et al., 2008; Cemin et al., 2017). However, there are also some reports on the effect of dietary Lys on meat quality. Increasing dietary Lys has been shown to increase final pH and decrease drip loss of broiler breast meat (Berri et al., 2008), and reducing dietary Lys has been shown to increase intramuscular fat in the longissimus dorsi muscles of finishing gilts (Katsumata et al., 2005). Conversely, there have been few reports on the relationship between dietary Lys and the meat taste.

In-mouth perception of taste is related to consumer satisfaction of meat (Resano et al., 2011). Various compounds contribute to meat flavor, including Glu, an important taste-active component in meat (Kato and Nishimura, 1987; Maga, 1994). Of the 5 basic tastes, Glu is "umami", which has also been described as "savory," "beefy," and "brothy" (Maga, 1994). Halpern (2000) reported that appropriate concentrations of Glu increase food palatability. For instance, the addition of Glu to chicken soup increased its intensity of umami, sweetness, and saltiness (Fujimura et al., 1996); thus,

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an increase in the free Glu content of chicken may improve its taste. However, dietary Glu is metabolized by intestinal cells during absorption, with only 5% of the Glu appearing in the portal blood (Reeds et al., 1996). Therefore, it is difficult to increase the free-Glu content of muscle by the feeding of Glu-rich feeds; instead, an indirect method is required. We recently found that the free-Glu concentration in chicken muscle can be increased by the feeding of a high- or low-Lys diet (Watanabe et al., 2015; 2017). These were the first reports on the relationship between dietary Lys and meat taste.

In the feeding of a high-Lys diet, the Lys degradation pathway contributed to the free-Glu production. Lys is mainly degraded by the saccharopine pathway in chickens (Grove and Roghair, 1971; Wang and Nesheim, 1972), and lysine α-ketoglutarate reductase (EC 1.5.1.8) is the primary enzyme of this pathway. Saccharopine and α-aminoadipic acid are the intermediate metabolites of the saccharopine pathway, and 1 mol of Glu is released when 1 mol of these intermediates is metabolized. Our recent study showed a significant increase in lysine α-ketoglutarate reductase mRNA abundance in chicken muscle from the feeding of a high-Lys diet, and muscular saccharopine and α-aminoadipic acid were also increased (Watanabe et al., 2015). These results suggest that the Lys degradation pathways in muscle were involved in the increase in free Glu in muscles. The feeding of a low-Lys diet to broilers also increased the free-Glu concentration in muscles (Watanabe et al., 2017). Although the mechanism is yet to be determined, this could be explained by a change in protein metabolism. Proteins are synthesized using free amino acids, and free amino acids are supplied by food and the degradation of preexisting proteins. Thus, a change in protein metabolism might affect the free amino acid concentrations in muscles. For instance, insulin-like growth factor (**IGF**) 1, which is a stimulator of protein synthesis, was significantly decreased in plasma of pigs fed a low-Lys diet (Katsumata et al., 2002). Also, we have previously shown that 3-methyl-histidine (**3M-His**), which is an indicator of skeletal muscle protein degradation, is significantly increased in chicken breast muscle by the feeding of a low-Lys diet (Watanabe et al., 2017). Therefore, a change in protein metabolism by low Lys feeding might induce an increase in muscle free amino acids including Glu.

In the present study, the effect of a low-Lys diet on the concentration of free Glu and other free amino acids in muscles was examined using broiler chickens. We also determined small-molecule metabolites. Finally, we investigated the effect of a low-Lys diet on the mRNA abundance of protein metabolism–related genes and their correlation with free Glu content to clarify mechanisms of Glu regulation.

MATERIALS AND METHODS

Animals

Ross strain female chicks were purchased at birth from a commercial hatchery (Onuma, Shibata, Japan). All chicks were housed in a brooder from day 0 to day 14, where they were kept warm, and in battery cages from day 15 to day 28. The room temperature was kept at 22 ± 2°C, and lighting was kept on a regular schedule (light for 15 h, 04:00 h to 19:00 h). All chicks were allowed free access to feed and water. Chicks were raised on a commercial diet containing 200 g/kg crude protein (**CP**) and 3.2 kcal/g metabolizable energy (**ME**). On day 28, chickens were allocated to 2 groups, (n = 10 per group) ensuring that the average weight of animals was the same between groups. During the experimental period, chickens were individually housed in cages. The study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Science Council of Japan. All the animal experiments were conducted in compliance with the protocol, which was reviewed by the Institutional Animal Care and Use Committee and approved by the President of Niigata University (permit number: #26 Niigata Univ. Res. 80-5).

Experimental Diets

The composition of the 2 experimental diets is shown in Table 1. The diets contained 100 or 90% of the Lys requirement, where 100% of the Lys requirement is 10.0 g/kg of the diet according to NRC (1994) guidelines. Both diets contained 3.2 kcal/g ME and 170 g/kg CP. Dietary amino acid, ME, vitamins, and mineral contents met the NRC requirements. Chickens in each group were fed their allocated diet for 10 consecutive days.

Sample Collection

At the end of the trial, feed intake was recorded for each individual, and the chickens were weighed. At 13:30 h, blood samples were taken from the wing vein, and chickens were killed by cutting the carotid arteries. Blood samples were collected in syringes treated with heparin solution (100 unit/mL), deproteinized with 3% sulfosalicylic acid, and the plasma stored at −20°C until determination of amino acid concentrations. Breast muscle was dissected to remove the epimysium, fat, vessels, and connective tissues and cut into small pieces. Muscle samples were frozen using liquid nitrogen and kept at −80°C until free amino acid measurements and real-time reverse transcriptase quantitative PCR (RT-qPCR) were performed.

Preparation of Muscle Extract

Muscles were homogenized separately in 10% perchloric acid using a high-speed homogenizer (Ultra Turrax T25 basic; IKA Werke, Staufen, Germany). The homogenate was centrifuged at 600 × g and kept at 4°C for 20 min, and then the supernatant was neutralized with 10% (w/v) potassium hydrate. After removal of the potassium crystals by filtration, the filtrate volume was adjusted to 50 mL using double-distilled water, and the samples were stored at −20°C until analysis.

Table 1. Composition of the experimental diets.

Ingredients (% of diet)	Dietary Lys level (% of requirement)[1]	
	100	90
Corn grain	74.00	73.50
Soybean meal	15.46	16.01
Fish white	5.00	5.00
Soybean oil	2.59	2.73
$CaCO_3$	0.43	0.43
$CaHPO_4$	1.36	1.35
NaCl	0.15	0.15
Vitamin and mineral premixture[2]	0.50	0.50
Lys-HCl	0.13	0.01
Arg	0.11	0.09
Met	0.12	0.12
Thr	0.12	0.11
Trp	0.01	
Ile	0.01	
Val	0.01	
Phe		
Total	100.00	100.00
Calculate analysis		
CP (%)	17.00	17.00
ME (kcal/g)	3.20	3.20
Essential amino acids (%)		
Lys	1.00	0.90
Arg	1.10	1.10
His	0.41	0.42
Ile	0.73	0.73
Leu	1.50	1.52
Met	0.38	0.38
Phe	0.75	0.76
Thr	0.74	0.74
Trp	0.18	0.18
Val	0.82	0.82

Abbreviations: CP, crude protein; ME, metabolizable energy.
[1]100% Lys requirement is 10.0 g/kg of diet (NRC, 1994).
[2]Content per kg of vitamin and mineral premixture: vitamin A 300,000 IU (retinyl acetate), vitamin D_3 40,000 IU, vitamin E 2,000 IU (DL-alpha-tocopheryl acetate), vitamin K_3 192 mg, thiamin nitrate 360 mg, riboflavin 720 mg, calcium δ-pantothenate 2,175 mg, nicotinamide 6,943 mg, pyridoxine hydrochloride 700 mg, biotin 30 mg, folic acid 110 mg, cyanocobalamin 2 mg, calcium iodinate 108 mg, MgO 198,949 mg, $MnSO_4$ 32,982 mg, $ZnSO_4$ 19,755 mg, $FeSO_4$ 43,521 mg, $CuSO_4$ 4,019 mg, and choline chloride 299,816 mg.

Measurement of Free Amino Acids

The concentrations of free amino acids in plasma and muscles were measured using an amino acid analyzer (JLC-500/V; JEOL, Tokyo, Japan). A multisegment tandem packed column (LC-500AC4016, Li type, 4 mm diameter × 160 mm; JEOL) was used. The detection wavelengths were 440 and 570 nm. Amino acids were detected using the ninhydrin method.

Metabolome Analysis

To screen the specific metabolism affected by reduced dietary Lys, the breast muscle metabolome was measured using a combined capillary electrophoresis and time-of-flight mass spectrometry (**CE-TOFMS**) system (Human Metabolome Technologies Inc., Tsuruoka, Japan). Samples in each group were pooled (n = 1), with each pooled sample weighing approximately 50 mg. The pooled samples were homogenized in 1,800 μL of 50% (v/v) acetonitrile solution (1,500 rpm × 120 s × 5 times), centrifuged at 2,300 × *g* for 5 min at 4°C, and the aqueous phase was passed through an ultrafiltration membrane (5-kDa cutoff filter; Ultrafree MC; Millipore, Darmstadt, Germany). Samples were then completely evaporated, and the residue dissolved in 50 μL of double-distilled water. The prepared samples were analyzed using an Agilent CE-TOFMS system (Agilent Technologies, Waldbronn, Germany) as described by Matsumoto et al., 2012. The extracted cationic and anionic metabolites were analyzed using a fused silica capillary (50 μm in diameter × 80 cm) with a commercial electrophoresis buffer (cation buffer solution, H3301-1001; anion buffer solution; H3302-1021; Human Metabolome Technologies Inc.). CE-TOFMS data processing was conducted, and peak information extracted using the automatic integration software MasterHands (Keio University, Tsuruoka, Japan), including ion mass/charge number (**m/z**), migration time for CE-TOFMS measurement (**MT**), and peak area (Sugimoto et al., 2009). The mass spectrometry scan range was 50 to 1,000 (m/z). Detected metabolites were identified based on m/z and their MT using the metabolite library maintained by Human Metabolome Technologies Inc. The relative area was calculated as follows: relative area = peak area/(peak area of internal control × sample volume). To calculate the fold-change between groups, the relative area of the Lys 90% group was divided by the relative area of the Lys 100% group.

RNA Isolation and Real-Time RT-qPCR Analysis

Total RNA was isolated from muscles using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA yield and quality were determined spectrophotometrically by the absorbance at the wavelengths of 260 and 280 nm, using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA synthesis was performed as described by Shibata et al. (2006). Briefly, first-strand cDNA was synthesized from 3 μg of total RNA using SuperScript II RNase H^- reverse transcriptase (Invitrogen) with oligo-dT primers (Invitrogen). After reverse transcription, analysis of cDNA abundance of IGF-1, IGF-2, myostatin, μ-calpain, m-calpain large subunit, caspase 3, cathepsin B, 20S proteasome C1 subunit, and 20S proteasome C2 subunit was performed by real-time RT-qPCR using LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics, Basel, Switzerland) and a LightCycler 1.5 instrument (Roche Diagnostics). Specific product amplification was checked by melting curve analysis. The primers for each gene were designed based on sequences reported in the study by Ibuki et al. (2013; 2014) and Watanabe et al. (2015). The primer sets used for PCR are shown in Table 2. The housekeeping gene *Gapdh* was used as a normalizing control. All data were analyzed using LightCycler Software version 3.5 (Roche Diagnostics).

Statistical Analysis

Data were analyzed using the SAS (version 9.4; SAS Institute, Cary, NC). Student *t* tests were used for growth

Table 2. Primers used in this study.[1]

Gene		Primer sequences	Accession number
IGF-1	Sense	5′-GCTGCCGGCCCAGAA-3′	NM_001004384
	Antisense	5′-ACGAACTGAAGAGCATCAACCA-3′	
IGF-2	Sense	5′-CCTGGCTCTGCTGGAAACC-3′	NM_001030342
	Antisense	5′-GAGAGGTCACGCTCTGACTTGA-3′	
Myostatin	Sense	5′-ATGCAGATCGCGGTTGATC-3′	NM_001001461
	Antisense	5′-GCGTTCTCTGTGGGCTGACT-3′	
μ-calpain	Sense	5′-CACACAAGGAGGCCGACTTC-3′	NM_205303
	Antisense	5′-TCCGCTGTGTCTGACTGCTT-3′	
20S proteasome C1 subunit	Sense	5′-TGAGGAACAAGGAGCCCATCT-3′	AB_001935
	Antisense	5′-TGCCCTTGTACTGATACACCAT-GT-3′	
20S proteasome C2 subunit	Sense	5′-CCAGTATCTCGTTTGGTGTCAC-TAC-3′	AF_027978
	Antisense	5′-CATAGCGCTGCGTTGGTATC-3′	
m-Calpain large subunit	Sense	5′-GTGGCTCGGTTTGCTGATG-3′	D_38026
	Antisense	5′-AATCAAGCACCGGACACAATT-3′	
Caspase 3	Sense	5′-GGAACACGCCAGGAAACTTG-3′	AF_083029
	Antisense	5′-TCTGCCACTCTGCGATTTACA-3′	
Cathepsin B	Sense	5′-GCTACTCGCCTTCCTACAAGGA-3′	U_18083
	Antisense	5′-GCGAGGGACACCGTAGGAT-3′	
GAPDH	Sense	5′-GTGGTGGCCATCAATGATCCC-3′	M_11213
	Antisense	5′-TGTTGCTGGGGTCACGCTCC-3′	

Abbreviations: GAPDH, glyceraldehydes-3-phospate dehydrogenase; IGF, insulin-like growth factor.
[1]The primers were designed based on sequence given in the study by Ibuki et al. (2013; 2014). All primers are shown 5′ to 3′.

performance, free amino acid concentrations, and mRNA abundance. Data are presented as means ± SEM. Student t tests were demonstrated by the TTEST procedures in SAS. The Pearson correlation coefficient was calculated to determine the relationship between free-Glu concentration and the mRNA abundance of protein metabolism–related genes in muscles. Pearson correlation coefficient was analyzed using the TTEST procedures in SAS. Differences and correlations of $P < 0.05$ were considered significant.

RESULTS

Growth Performance

The growth performance of chickens fed the Lys 90% diet for 10 D is shown in Table 3. Total Lys intake was decreased in chickens fed the Lys 90% diet ($P < 0.05$) compared with those fed the Lys 100% diet. Feed intake, body weight gain, and feed efficiency were not significantly different between the groups.

Free Amino Acids in Plasma and Muscle

The free amino acid concentrations in plasma are shown in Table 4. The concentration of plasma Lys ($P < 0.01$) and Met ($P < 0.05$) was decreased in the Lys 90% group, whereas that of plasma His ($P < 0.01$) was increased in the Lys 90% group ($P < 0.01$) compared with the Lys 100% group. There were no significant differences between groups for other plasma free amino acids.

The free amino acid concentrations in muscles are shown in Table 5. The muscle free Lys concentration was decreased in the Lys 90% group ($P < 0.01$) compared with that in the Lys 100% group. The concentration of muscle free Glu was increased in the Lys 90%

Table 3. Effect of dietary lysine level on the growth performance of broilers.[1]

Growth performance and tissue weight	Dietary Lys level (% of requirement) 100		90		Significance
Initial body weight (g)	1,109.0	±31.9	1,107.6	±30.2	NS
Body weight gain (g/10 D)	811.5	±33.8	812.5	±25.6	NS
Feed intake (g/10 D)	1,467.1	±50.8	1,466.0	±40.3	NS
Feed efficiency (g body weight gain/g intake)	0.552	±0.008	0.554	±0.006	NS
Lys intake (g/10 D)	14.7	±0.5	13.2	±0.4	*
Breast muscle: body weight (g/kg BW)	143.5	±3.3	140.2	±2.1	NS
Abdominal fat: body weight (g/kg BW)	17.2	±1.8	16.3	±1.2	NS
Liver: body weight (g/kg BW)	25.5	±1.1	25.9	±0.9	NS

*$P < 0.05$.
Abbreviations: BW, body weight; NS, not significant.
[1]Values of Lys 100% and Lys 90% groups are means ± SEM for 10 chickens.

Table 4. Effect of dietary lysine level on free amino acid concentrations in plasma of broilers (nmol/mL plasma).[1]

Amino acids	Dietary Lys level (% of requirement) 100	90	Significance
Tau	599.7 ± 86.4	584.4 ± 61.8	NS
Asp	56.1 ± 2.0	54.5 ± 5.6	NS
Thr	1,076.8 ± 51.8	1,076.9 ± 48.8	NS
Ser	1,131.3 ± 69.1	981.1 ± 70.0	NS
Glu	508.3 ± 22.1	447.9 ± 38.2	NS
Gln	836.8 ± 43.7	746.4 ± 57.9	NS
Gly	887.6 ± 74.0	871.1 ± 52.3	NS
Ala	1,264.5 ± 115.8	1,135.7 ± 96.2	NS
Val	208.1 ± 12.8	215.3 ± 10.3	NS
Met	99.6 ± 5.6	82.4 ± 3.4	*
Ile	103.8 ± 6.9	103.4 ± 5.7	NS
Leu	302.9 ± 12.2	303.0 ± 16.2	NS
Tyr	286.0 ± 24.8	251.2 ± 22.2	NS
Phe	145.3 ± 7.2	150.5 ± 7.2	NS
His	146.8 ± 14.3	204.2 ± 15.2	**
Lys	186.6 ± 13.8	102.7 ± 4.9	**
Arg	639.2 ± 42.2	549.2 ± 37.7	NS
3M-His	22.6 ± 1.7	26.1 ± 1.2	NS

$^{**}P < 0.01$; $^{*}P < 0.05$.
Abbreviation: NS, not significant.
[1]Values of Lys 100% and Lys 90% groups are means ± SEM for 10 chickens.

Table 5. Effect of dietary lysine level on free amino acid concentrations in muscle of broilers (μg/g muscle).[1]

Amino acids	Dietary Lys level (% of requirement) 100	90	Significance
Tau	259.8 ± 64.2	193.1 ± 24.9	NS
Asp	66.2 ± 5.6	62.4 ± 4.4	NS
Thr	132.0 ± 6.0	198.0 ± 17.5	**
Ser	162.9 ± 10.7	189.0 ± 16.1	NS
Glu	136.4 ± 9.1	207.0 ± 9.7	**
Gln	268.3 ± 23.1	363.0 ± 32.5	*
Gly	205.5 ± 25.6	472.1 ± 63.6	**
Ala	194.7 ± 10.9	237.4 ± 23.4	NS
Val	11.2 ± 0.8	23.9 ± 1.9	**
Met	8.9 ± 1.0	10.7 ± 1.4	NS
Ile	6.3 ± 0.9	11.7 ± 0.7	**
Leu	20.2 ± 1.2	31.7 ± 2.1	**
Tyr	55.4 ± 5.2	75.4 ± 5.1	**
Phe	18.3 ± 1.6	29.7 ± 2.6	**
His	2.2 ± 0.4	12.0 ± 2.5	**
Lys	10.1 ± 0.5	6.2 ± 0.9	**
Arg	92.3 ± 9.0	113.2 ± 9.1	NS
3M-His	4.1 ± 0.5	6.0 ± 0.5	*

$^{**}P < 0.01$; $^{*}P < 0.05$.
Abbreviation: NS, not significant.
[1]Values of Lys 100% and Lys 90% groups are means ± SEM for 10 chickens.

group ($P < 0.01$) by a factor of 51.8%. Muscle free Thr, Gly, Val, Ile, Leu, Tyr, Phe, His, Gln, and 3M-His concentrations were also increased in chickens fed the Lys 90% diet ($P < 0.05$). Other muscle free amino acids showed no significant differences between groups.

Metabolome Analysis

A total of 133 metabolites were identified in the breast muscle. We divided these into 3 groups on the basis of the fold-change in the Lys 90% group compared with the Lys 100% group, as follows: upregulated (>1.5-fold), downregulated (<0.67-fold), and unchanged (0.67 to 1.5-fold). Using these criteria, 22 metabolites were upregulated and 24 were downregulated in the Lys 90% group, as shown in Table 6. The upregulated metabolites included amino acids and metabolic intermediates of amino acids, metabolites of glycolysis and the citric acid cycle, and nucleoside triphosphate. The downregulated metabolites included Lys-related metabolites, such as β-Ala-Lys, saccharopine, N6-acetyllysine, cadaverine, pipecolic acid, and nucleoside monophosphate.

mRNA Abundance of Protein Metabolism–Related Genes in Muscles

Results for mRNA abundance of protein metabolism–related genes are shown in Table 7. All data are expressed as a percentage of the Lys 100% group. mRNA abundance of μ-calpain, caspase 3, and 20S proteasome C2 subunit was significantly increased in the Lys 90% group compared with the Lys 100% group ($P < 0.01$ except μ-calpain, $P < 0.05$). mRNA abundance of other protein metabolism–related genes showed no significant differences between groups.

Pearson Correlation Between Free Glu and mRNA Abundance

Results for the Pearson correlation between free Glu concentration and mRNA abundance of protein metabolism–related genes in muscles are shown in Figure 1. The muscle free Glu concentration was correlated with the mRNA abundance of μ-calpain ($r = 0.74$, $P < 0.01$), caspase 3 ($r = 0.69$, $P < 0.01$), 20S proteasome C2 subunit ($r = 0.65$, $P < 0.01$), and cathepsin B ($r = 0.52$, $P < 0.05$). mRNA abundance of other protein metabolism–related genes was not significantly correlated with muscle free Glu concentration (data not shown).

DISCUSSION

Glu is a taste-active component of meat, contributing a "umami" flavor (Kato and Nishimura, 1987; Maga, 1994). An increase in the free Glu content of meat enhances taste (Imanari et al., 2008; Watanabe et al., 2017) and may thereby improve meat taste. However, dietary Glu does not proportionally affect portal blood Glu because the majority of dietary Glu is metabolized by intestinal cells during absorption in pigs (Reeds et al., 1996). Glu metabolism in chicken intestinal cells is slightly different from mammals because of the deficiency in ornithine aminotransferase (Wu et al., 1995). However, Glu is the preferred fuel for respiration in chicken intestinal cells (Watford et al., 1979), and Glu supplementation in the diet did not increase the free Glu concentration in the plasma (Maruyama et al., 1976). Therefore, it is difficult to increase the free Glu content of muscle by feeding with Glu-rich feeds. We have previously reported that the feeding of a high Val, Ile, or CP diet increases the free Glu content in breast muscle of broilers (Imanari et al.,

Table 6. Effect of dietary lysine level on metabolites in breast muscle in broilers.[1]

Compound name[2]	m/z	MT	Relative area		Fold-change (vs. Lys 100%)
			Lys 100%	Lys 90%	
Upregulated					
UTP	482.96	12.48	ND[3]	2.4E-03	<1
Diethanolamine	106.09	7.23	ND	4.5E-04	<1
ATP	505.99	11.66	6.9E-03	4.6E-02	6.58
His	156.08	6.92	6.1E-03	2.0E-02	3.31
3-Methylhistidine	170.09	7.09	6.7E-03	2.1E-02	3.14
GTP	521.99	11.36	5.5E-04	1.2E-03	2.25
5′-Deoxy-5′-methylthioadenosine	298.09	9.36	6.6E-05	1.4E-04	2.05
S-Adenosylmethionine	399.14	6.75	2.3E-03	4.7E-03	2.00
Cystathionine	223.07	9.21	5.5E-04	1.1E-03	1.93
Imidazolelactic acid	157.06	8.20	1.8E-04	3.3E-04	1.83
Gly	76.04	7.83	3.0E-01	5.4E-01	1.82
ADP	426.02	10.86	1.2E-02	2.1E-02	1.76
Ile	132.10	9.47	1.9E-02	3.3E-02	1.74
Sarcosine	90.05	8.84	7.5E-03	1.3E-02	1.70
Succinic acid	117.02	20.97	4.3E-03	7.0E-03	1.60
Glucose 1-phosphate	259.02	10.05	1.7E-02	2.7E-02	1.60
Homoserinelactone	102.06	6.75	4.6E-04	7.2E-04	1.57
Glucose 6-phosphate	259.02	9.78	3.7E-01	5.7E-01	1.55
Val	118.09	9.31	3.5E-02	5.4E-02	1.55
Arg	175.12	6.75	8.7E-02	1.3E-01	1.53
Citric acid	191.02	26.40	2.9E-03	4.5E-03	1.53
Histamine	112.09	4.60	1.4E-03	2.1E-03	1.50
Downregulated					
Urea	61.04	18.08	2.0E-02	ND	1 <
Homoarginine	189.13	6.64	1.0E-03	ND	1 <
β-Ala-Lys	218.15	6.34	5.8E-04	ND	1 <
Saccharopine	277.14	9.91	1.5E-04	ND	1 <
Gly-Leu	189.12	8.95	3.8E-04	ND	1 <
Ala-Ala	161.09	8.65	4.4E-04	ND	1 <
CMP	322.04	9.53	1.2E-03	1.9E-04	0.16
Guanidoacetic acid	118.06	7.76	4.0E-03	8.0E-04	0.20
β-Ala	90.05	6.94	1.2E-01	2.7E-02	0.23
UMP	323.03	9.72	3.7E-03	1.0E-03	0.28
N^2-Succinylornithine	233.11	9.51	1.5E-03	4.5E-04	0.31
GMP	362.05	9.07	3.1E-03	1.1E-03	0.34
3-Guanidinopropionic acid	132.08	7.56	1.1E-02	4.1E-03	0.36
Adenosine	268.10	9.18	2.9E-04	1.4E-04	0.49
AMP	346.06	9.17	3.1E-02	1.6E-02	0.52
Ribulose 5-phosphate	229.01	10.91	2.4E-03	1.3E-03	0.53
N^6-Acetyllysine	189.12	9.11	8.2E-04	4.4E-04	0.53
Carnosine	227.11	6.36	1.0E+00	5.4E-01	0.54
Inosine	269.09	16.90	1.7E-02	9.7E-03	0.56
Cadaverine	103.12	4.79	9.6E-05	5.6E-05	0.58
Spermine	203.22	4.33	1.6E-03	9.5E-04	0.60
Pipecolic acid	130.09	9.53	2.9E-03	1.8E-03	0.62
Ascorbic acid	175.02	8.38	1.2E-03	7.6E-04	0.62
Isobutyrylcarnitine	232.15	8.85	3.4E-04	2.2E-04	0.65

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; CMP, cytidine monophosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; UMP, uridine monophosphate; UTP, uridine triphosphate.

[1]All samples in each group were pooled (n = 1).

[2]Compound name based on mass-to-charge ratio (m/z) and migration time (MT) by using the metabolite library maintained by Human Metabolome Technologies (Tsuruoka, Japan).

[3]ND, not detected in Lys 100% or Lys 90% group.

2008; Kobayashi et al., 2011), indicating that dietary amino acid and protein levels regulate the free Glu concentration in muscles. In addition, we recently showed that the feeding of a high- or low-Lys diet increases the muscle free Glu concentration of broilers (Watanabe et al., 2015; 2017).

The goal of the present study was to elucidate the mechanisms by which the feeding of a low-Lys diet increases free Glu in chicken muscles. This study provides new insight into amino acid metabolism in skeletal muscle and may lead to a more efficient method for increasing muscular free Glu to improve meat taste. Our results show that the muscle concentration of free Glu, as well as that of various other amino acids, was significantly increased in the Lys 90% group compared with the Lys 100% group. However, the plasma concentration of free amino acids, with the exception of Met, Lys, and His, was no different between groups. Therefore, muscle free amino acids that accumulated from the feeding of a low-Lys diet were not derived from plasma.

A metabolome analysis was performed to further investigate the metabolic changes in muscle induced by the low-Lys diet. Results showed that saccharopine and pipecolic acid, which are intermediates in the Lys degradation

Table 7. Effect of dietary lysine level on mRNA abundance of factors of protein metabolism (% of Lys 100% group) in broiler muscles.

Factors[1]	Dietary Lys level (% of requirement)		
	100	90	Significance
Factors of protein synthesis			
IGF-1	100.0 ± 9.1	110.4 ± 7.1	NS
IGF-2	100.0 ± 17.1	87.1 ± 9.1	NS
Myostatin	100.0 ± 15.3	91.3 ± 3.7	NS
Factors of protein degradation			
μ-Calpain	100.0 ± 5.3	121.2 ± 5.8	*
m-Calpain large subunit	100.0 ± 10.3	105.2 ± 15.0	NS
Caspase 3	100.0 ± 9.5	186.3 ± 19.4	**
Cathepsin B	100.0 ± 7.6	111.6 ± 5.3	NS
20S proteasome C1 subunit	100.0 ± 18.2	106.7 ± 16.0	NS
20S proteasome C2 subunit	100.0 ± 5.5	123.3 ± 4.0	**

**$P < 0.01$; *$P < 0.05$.

Abbreviations: IGF, insulin-like growth factor; NS, not significant.

[1]Abundance of mRNA in the breast muscle of chicks fed the Lys 100% or Lys 90% group expressed as % of Lys 100% group. Values of Lys 100% and Lys 90% groups are means ± SEM for 10 chickens.

pathway, were decreased in the Lys 90% group. We have previously shown that saccharopine and pipecolic acid are increased by the feeding of a high-Lys diet and suggested that this change plays a key role in the increase in muscle free Glu from high Lys feeding (Watanabe et al., 2015). The mechanisms underlying the increase in muscle free Glu thus appear to differ between low-Lys and high-Lys feeding conditions. Of the 22 metabolites upregulated from low-Lys feeding, 9 were amino acids, suggesting increased free amino acids to be a major metabolic change that occurs during low-Lys feeding. Also, metabolites of glycolysis and the citric acid cycle and nucleoside triphosphate were increased in the Lys 90% group compared with the Lys 100% group. In contrast, nucleoside monophosphate was decreased in the Lys 90% group, suggesting that adenosine triphosphate synthesis by glycolysis, and the citric acid cycle was stimulated by low Lys feeding. In addition, carnosine and ascorbic acid were decreased in the Lys 90%. Carnosine is a naturally occurring skeletal muscle dipeptide comprised of β-alanine and histidine

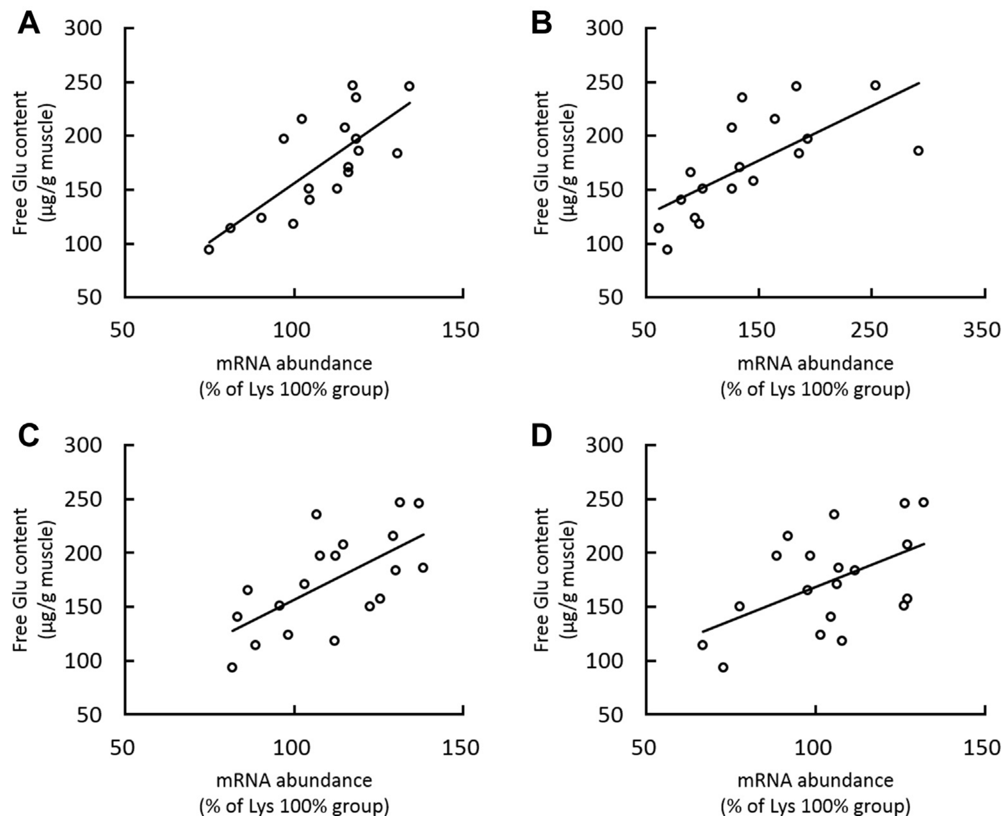

Figure 1. The Pearson correlation of mRNA abundance of (A) μ-calpain, (B) caspase 3, (C) 20S proteasome C2 subunit, and (D) cathepsin B with free glutamate (Glu) content in broiler muscles. (A) $r = 0.74$, $P < 0.01$; (B) $r = 0.69$, $P < 0.01$; (C) $r = 0.65$, $P < 0.01$; (D) $r = 0.52$, $P < 0.05$.

(Crush, 1970), and it reduces oxidative stress by its peroxyl radical-trapping ability (Kohen et al., 1988). An increase in carnosine in broiler meat improves its drip loss (Kralik et al., 2018), 2-thiobarbituric acid reactive substances values (Hu et al., 2009; Kralik et al., 2018), and shear force value (Hu et al., 2009). Ascorbic acid is also an antioxidant that partly improves growth performance and meat quality, such as pH and color in broilers under heat stress (Imik et al., 2012). Therefore, the decrease in these antioxidants due to the reduction in dietary Lys may induce a negative effect on the meat quality. The metabolome analysis reveals some metabolic changes induced by the feeding of a low-Lys diet. However, this analysis could not address all the factors contributing to Glu production, and further study is required.

The increase in muscle free amino acids from the feeding of a low-Lys diet might be explained by a change in protein metabolism in muscle because free amino acids are mainly derived from the degradation of preexisting proteins (Ohsumi, 2006), and proteins are synthesized from free amino acids. Protein metabolism is affected by many nutritional factors including energy status (Proud, 2007), type of protein (Morifuji et al., 2005; Wilkinson et al., 2007), amino acids (Borsheim et al., 2002; Kadowaki and Kanazawa, 2003), and Leu (Nagasawa et al., 2002; Norton and Layman, 2006). In a recent study, Sato et al. reported that Lys and saccharopine both affect protein metabolism by suppressing autophagic-proteolysis through Akt (Sato et al., 2013; 2015). Here, we show that the muscle 3M-His content was significantly increased in the Lys 90% group. 3M-His in excreta is indicative of the rate of myofibrillar protein degradation in chicken skeletal muscle (Hayashi et al., 1985); therefore, an increase in muscle free 3M-His content may suggest an increase in the rate of myofibrillar protein degradation. Together, these results suggest that dietary Lys content is an important factor in protein metabolism.

To investigate changes in muscle protein metabolism induced by low Lys feeding in broilers, we measured the mRNA abundance of protein metabolism–related genes. Our results show that the mRNA abundance of μ-calpain, caspase 3, and 20S proteasome C2 subunit in muscle was significantly increased in the Lys 90% group compared with that in the Lys 100% group. These results suggest that protein degradation is enhanced by the feeding of a low-Lys diet. Skeletal muscle amino acid reserves are the only storage depot that can experience losses without compromising the ability to sustain life (Miller, 2007). Therefore, skeletal muscle might respond to a decrease in the body's Lys concentration with protein degradation. Moreover, the mRNA abundance of μ-calpain, caspase 3, cathepsin B, and 20S proteasome C2 subunit was significantly correlated with free Glu concentration in muscles. Thus, protein degradation was related to the increase in free Glu in chicken muscles after the feeding of a low-Lys diet. Conversely, IGF-1 and IGF-2 showed no difference between groups, suggesting that regulation of protein synthesis by IGF-1 and IGF-2 did not contribute to the increase in free Glu in muscle after the feeding of a low-Lys diet.

In conclusion, we found that reducing dietary Lys significantly increased the content of free Glu and other free amino acids in chicken muscle. The mRNA abundance of protein degradation enzymes also significantly increased with low-Lys diet feeding and correlated with muscle free Glu concentration. These results suggest that protein degradation was induced by the reduction of dietary Lys, which released free amino acids that were stored in muscles. In the present study, growth performance did not decrease as a result of this protein degradation in muscle; however, productivity may be affected by Lys restriction. In future studies, we will determine the optimal Lys restriction level and feeding duration based on the enhancement of meat taste by the increase in Glu and the chickens' growth performance. In addition, results of metabolome analysis could not clearly have stated because metabolome analysis was performed on a pooled sample in this study. Thus, we will confirm the reproducibility of the metabolome analysis by individual samples. Especially, we will analyze the effect of the low-Lys diet on the antioxidants' concentration in the meat. Moreover, the effects of reduction in dietary Lys on the meat quality, such as pH, drip loss, 2-thiobarbituric acid reactive substances values, and shear force value will be examined. Based on these results, we will test antioxidant supplementation in the low-Lys treatment.

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